EXHIBIT E

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Mechanistic and Statistical Insight into the Large Carcinogenesis Bioassays on *N*-Nitrosodiethylamine and *N*-Nitrosodimethylamine

James A. Swenberg,[1] David G. Hoel, and Peter N. Magee

Department of Environmental Sciences and Engineering, The University of North Carolina, Chapel Hill, North Carolina 27599 [J. A. S.]; National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 [D. G. H.]; and 12 Lancaster Road, Wimbledon, London, SW195DD, England, United Kingdom [P. N. M.]

This issue of *Cancer Research* contains three articles describing detailed carcinogenesis bioassays on NDEA[2] and NDMA and related chemicals that were conducted at the British Industrial Biological Research Association by Paul Brantom and analyzed at the ICRF Cancer Studies Unit under the direction of Richard Peto (1–3). They provide the most detailed dose-response data for any carcinogen in rats and utilize advanced statistical methods to evaluate the data. The purpose of this commentary is to draw together the present mechanistic understanding on these carcinogens with the detailed bioassay data in order to help appreciate critical interactions and areas for future study.

The remarkable differences between the rates at which particular types of human cancer occur in different parts of the world indicate that environmental factors must play an important role in their causation (4). These environmental factors include chemicals, radiation, and viruses, acting individually or in combination. Occupational exposure to chemicals in the workplace has been recognized as the cause of some human cancers for many years. Opinions have differed on the extent to which occupational exposure contributes to the totality of human cancer death, and Doll and Peto (4) concluded in 1981 that, in the United States, this might only be about 4%. The same authors noted that the use of tobacco is a major cause of human cancer, accounting then for about 30% of the United States total. Presumably, almost all of these tobacco induced cancers are due to chemical carcinogens. Various other environmental factors were reviewed, and a major fraction of the total incidence of human cancer, about 35%, was attributed to various rather nonspecific dietary factors, some of which presumably involve chemical carcinogens (4). Thus, it is likely that a large proportion of human cancer is due to chemical carcinogens.

Environmental chemicals may be man-made, such as the large number of old and new compounds produced by the chemical and pharmaceutical industries. These include industrial chemicals, drugs, food additives, pesticides, and herbicides. Also, as pointed out by Ames *et al.* (5), many naturally occurring chemical products of plants, fungi, or other microbial sources may be extremely important.

Recognition of the presence of chemical carcinogens in the environment to which human beings are exposed by ingestion, inhalation, or other routes has resulted, over the years, in the development of increasingly sophisticated tests for identifying potential human carcinogens, including whole animal bioassays and an array of *in vitro* tests. Positive findings in such tests have led to the classification of a large number of chemicals as being carcinogenic in animals. In contrast, the number of chemicals that have thus far been shown convincingly to be carcinogenic in humans is relatively small (probably not more than 50), according to the criteria of the International Agency for Research on Cancer (6).

The implications of positive results in animal carcinogenicity bioassays, with or without supporting data from *in vitro* or other short-term tests, have been discussed and debated extensively. Regulatory agencies in different countries have developed policies for the interpretation of these tests with a broad consensus that chemicals found to be carcinogenic in properly conducted laboratory studies should be regarded being potentially carcinogenic for humans. However, problems concerning extrapolation from high to low doses, the shape of the dose-response curve at low doses, and the existence of no-effect threshold exposures are unresolved.

There are two broad types of carcinogenic agents, those that have the capacity to induce structural changes in cellular DNA and those that do not (7). Compounds in the first category, sometimes described as genotoxic, probably act via mutational changes in target cells arising from interaction of the activated form of the carcinogen with DNA. Theoretically, such changes in a single somatic cell could result in the initiation of unrestricted cellular replication, leading to cancer through multiple subsequent steps. The carcinogenic response to such chemicals would not be expected to show a threshold, although there might be a nonlinear dose response. On the other hand, carcinogens with no capacity for inducing structural DNA changes, including tumor promoters, could act by other mechanisms compatible with the existence of a threshold.

Both as a guide to the discussions of dose-response relationships by regulatory agencies and as a guide to some of the basic mechanisms of carcinogenesis, reliable evidence about dose-response relationships in animals would be helpful, and one of the largest such studies ever done is now fully reported (1–3). The statistical studies of Peto and Gray, based on the experimental work by Brantom and Grasso, investigate the dose-response relationships of two carcinogenic nitrosamines over a dose range larger than used hitherto with any chemical carcinogen. These studies have involved large numbers of animals and, because they have utilized continual exposure throughout the life span of the rodents, they have demonstrated carcinogenic effects at unusually low exposures.

The nitrosamines used in the BIBRA studies, together with various other *N*-nitroso compounds, form a large group of typically genotoxic carcinogens (8, 9). Nitrosamines have been found to be carcinogenic in over 40 animal species and one or more of the compounds has induced tumors in almost every organ in rodents, the organ specificity often varying with the chemical structure of the compound. They are metabolically activated by the cytochrome P-450 superfamily of microsomal mixed function oxidases (10) and, with those nitrosamines studied thus far, the biologically active intermediates (ultimate carcinogens) have been alkylating agents, probably alkyldiazonium ions, which react with nucleophilic sites in cellular macromolecules, including DNA. The mechanisms of repair of the

[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethylamine; O^{6}MG, *O*^{6}-methylguanine; O^{6}EG, *O*^{6}-ethylguanine; N7MG, 7-methylguanine; O^{4}MT, *O*^{4}-methylthymidine; O^{6}AT, *O*^{6}-alkylguanine alkytransferase; O^{4}ET, *O*^{4}-ethylthymidine; BIBRA, British Industrial Biological Research Association; NTP, National Toxicology Program.

alkyl lesion in DNA have been extensively studied *in vitro* and *in vivo*. Activated *ras* oncogenes, with the expected type of mutation for this carcinogen in the twelfth codon, have been found in rat mammary tumors induced by *N*-methylnitrosourea (11).

Generally similar mechanistic patterns have been found with other genotoxic carcinogens, including polycyclic aromatic hydrocarbons and aromatic amines, suggesting that conclusions about nitrosamines may be generalized to other genotoxic chemical carcinogens. Thus, the work of Peto and his colleagues provides a unique source of data for analysis of dose-response relationships in chemical carcinogenesis and of the mechanisms that drive these responses, such as DNA replication and DNA repair. It also includes very important and innovative advances in biostatistical methodology.

The BIBRA (1, 2) studies on NDMA and NDEA represent the only carcinogenesis studies that have extensively evaluated dose-response relationships for tumor induction over 2.5 orders of magnitude. The ED_{01} study on 2-acetylaminofluorine utilized larger numbers of mice but covered doses of less than 1 order of magnitute. Investigations over at least a few orders of magnitude may clarify dose-response relationships and improve our ability to extrapolate risks from the high doses normally used in carcinogenesis bioassays to the lower exposures usually associated with environmental exposures. Since many of the mechanistic processes involved in chemical carcinogenesis are either saturable or inducible, dose-related differences in the amount of the carcinogen that reaches the DNA are likely (13, 14). Understanding the molecular dosimetry, *i.e.*, the relationship between the administered dose and the amount of each type of DNA adduct present on the DNA, along with the efficiency of each of the DNA adducts to cause mutations and the extent of cell proliferation present under each dosing scenario should greatly improve our understanding of causal factors in carcinogenesis and may make possible more accurate predictions of risk (15).

Carcinogenic Mechanisms of NDEA and NDMA

NDEA and NDMA are simple alkylating agents that require metabolic activation in order to generate their ethylating and methylating electrophiles. The major pathway for this biotransformation is thought to involve P450IIE1 (10), an enzyme with its greatest activity in the centrilobular hepatocytes (16, 17). In addition to P450IIE1, other isozymes can metabolize NDEA.[3] In the large bioassays reported in this issue (1, 2), NDEA induced a clear dose-related incidence of esophageal tumors in Colworth rats, wereas NDMA was devoid of such activity. This is consistent with tissue and chemical specific differences in DNA alkylation, as viewed by immunohistochemistry (18). The target site differences observed between NDMA and NDEA suggest that P450s other than P450IIE1 are responsible for esophageal metabolism of NDEA.

The electrophiles generated by NDEA and NDMA induce similar DNA adducts; however, the proportion of adducts at each base differs (19). NDMA methylates the DNA at many different sites. The extent of methylation is greatest at the N-7 position of guanine, followed by the O^6 position of guanine and the N-3 position of adenine. Of these three major sites of methylation, it is O^6MG that has the strongest correlation with carcinogenesis. O^6MG is formed at one-tenth the amount of N7MG but is highly efficient at causing G → A transitions. N7MG does not cause base pair mismatches with any degree of efficiency but does undergo chemical depurination leading to apurinic sites. If DNA replicates past an apurinic site, a G → T transversion can result. A second promutagenic adduct formed by NDMA is O^4MT. This adduct causes T → C transitions but is only formed at 1/100 the amount of O^6MG. NDEA produces the same ethylated adducts; however, the proportions are quite different. Ethylated phosphotriesters are the most common adduct but have not been shown to cause mutations. *N*-7-Ethylguanine represents about 10% of the DNA adducts, while O^6EG comprises 7%. The pyrimidines undergo more extensive alkylation of oxygens by ethylating agents than by methylating agents (20, 21). The *O*-alkylated pyrimidines are promutagenic and are repaired less well than O^6EG.

Major advances (reviewed in Ref. 22) have been made in understanding the repair of O^6-alkylguanine since the BIBRA studies were initiated. In mammalian cells, O^6MG is repaired by O^6-alkylguanine alkyltransferase (O^6AT), a protein that transfers the methyl group from O^6MG to an alkyl acceptor cysteine on the repair protein. This converts O^6MG to guanine and inactivates the molecule of O^6AT. The process is most efficient for methyl adducts and less efficient for ethyl adducts. Tissues and cell types differ both in the constitutive amount of O^6AT and in their ability to resynthesize the protein. Prominent species differences also exist, with humans having about 10-fold greater repair capacity than rats. Treatment of rats with a variety of agents, including NDMA and NDEA, results in an induction of O^6AT in liver, further enhancing its ability to repair O^6-alkylguanine. As will be discussed below, this has dramatic effects on the molecular dosimetry of DNA adducts during chronic exposure to NDMA and NDEA.

Our understanding of molecular dosimetry for the two nitrosamines studied by Peto *et al.* (1, 2) is greatest for NDEA in rat liver. Although O^6EG is the major promutagenic adduct formed in liver DNA of NDEA exposed rats, it is rapidly repaired. The exact mechanism of this repair *in vivo* is not known, but it is likely that O^6AT is involved. O^6EG was present in hepatocyte DNA at its highest concentration on the first day of exposure to NDEA and decreased thereafter (23). In contrast, O^4ET accumulated over the first 28 days of NDEA exposure, reaching concentrations 50-fold greater than that of O^6EG. Thus, the relative molecular dose of these two promutagenic adducts during chronic administration of NDEA differed 200-fold from what would have been predicted by initial alkylation rates. More recently, Boucheron *et al.* (24) reported an extensive molecular dosimetry study on O^4ET in livers of rats exposed for 1 to 70 days to drinking water containing concentrations of NDEA ranging from 0.4 to 100 ppm. The protocol was similar to that of Peto *et al.* (1, 2) and overlapped in exposure range. Hepatic O^4ET concentrations increased rapidly during the first 7 days of exposure and by 7 to 28 days accumulated to apparent steady-state concentrations that were linearly related to drinking water concentrations at all but the highest dose. The less than linear response at 100 ppm was primarily due to increased loss of cells due to excessive cytotoxicity (see below). Data on lower exposures suggested that repair of O^4ET also may be a saturable process, in that it took longer to reach the apparent steady state. Further delineation of this aspect will require the development of more sensitive assays for O^4ET. Such a saturable DNA repair mechanism could be an important factor in the marked nonlinearity in tumor response demonstrated by Peto *et al.* (1, 2). The BIBRA studies examined exposures

[3] C. S. Yang, personal communication.

extending slightly more than one order of magnitude lower than the molecular dosimetry studies of Boucheron *et al.* (24).

Much less is known about the molecular dosimetry of DNA adducts in other cell types of NDEA exposed rat livers. In the BIBRA (1, 2) studies the majority of liver tumors induced were of hepatocellular origin; however, other types were also produced. The only data on DNA adducts in specific cell types that are available are for rats exposed to 40 ppm NDEA for 1 to 77 days (23). This is higher than any of the doses used in the BIBRA studies. The concentration of O^4ET in nonparenchymal cells (which includes endothelial cells and Kuppfer cells and would correspond to the mesenchymal and Kuppfer cell tumors in the BIBRA studies) was approximately one-half that present in hepatocytes. O^4ET concentrations in nonparenchymal cells were 4- to 20-fold greater than O^6EG concentrations, so if O^4ET is an important molecular dosimeter for carcinogenesis it is somewhat surprising that more tumors of nonparenchymal cells were not seen.

The repair of O^4ET and that of O^4MT have been compared (25). Whereas O^4ET (produced by NDEA) has a $t_{1/2}$ of ~11 days in rat liver, O^4MT is repaired much more rapidly. When rats were exposed to either dimethylhydrazine (25) or 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (26) the $t_{1/2}$ for O^4MT was ~20 h, while rats treated with NDMA at 20 mg/kg repaired O^4MT with a $t_{1/2}$ of ~38 h (27). No data are available for the accumulation of O^4MT in liver DNA of rats chronically treated with NDMA. Richardson *et al.* (25) did show that O^4MT accumulated in hepatocyte DNA of rats exposed to dimethylhydrazine for up to 28 days, such that this minor promutagenic adduct attained apparent steady-state concentrations similar to those of O^6MG even though it is formed at only 1/100 the amount. This difference most likely results from more efficient repair of O^6MG.

Several studies have been conducted on the formation of DNA adducts in NDMA exposed rats, mice, and hamsters and these may be relevant to the present dose-response studies in those species (1–3). Single doses of NDMA induce kidney, but not liver tumors in rats. Such dosing regimens induce DNA alkylation in both liver and kidney, but O^6MG is more persistent in kidney DNA (28). The distribution and extent of alkylation were also dependent on the dose and route of administration. When NDMA was administered in large doses, liver and kidney DNA alkylation occurred regardless of route. When small doses of NDMA were administered p.o., DNA alkylation occurred only in liver, while i.p. administration resulted in both hepatic and renal alkylation (29, 30). This difference was attributed to first pass clearance of low doses of NDMA by the liver following oral exposure. The same phenomenon provides a reasonable explanation for the lack of kidney tumors in the BIBRA (1, 2) studies. NDMA does not alkylate esophageal DNA (18).

An additional observation of Pegg and Hui (30) was that the dose response for N7MG was different than that of O^6MG in liver DNA. At high doses of NDMA (>1 mg/kg), the ratio of N7MG to O^6MG was approximately 10, while at lower doses this ratio was 100 or greater. The change in the N7MG/O^6MG ratio was due to increased removal of O^6MG by O^6AT at low doses. At high doses of NDMA, O^6AT becomes saturated and the ratio of N7MG/O^6MG depends on the amounts of each adduct that are chemically formed. Thus, there is a nonlinearity in the molecular dose of O^6MG present in liver DNA between high and low doses of NDMA that corresponds closely with the change in the exponent for the median time to tumor in the equation

$$(\text{Dose rate}) \times (\text{median})^n = \text{Constant}$$

This exponent is 2.3 for doses above 1 ppm and 1.0 at doses below this. While this correlation supports the concept that molecular dose is a critical factor in carcinogenesis, much additional research is needed. Data following multiple exposures to NDMA demonstrate that O^6AT is clearly induced and that O^6MG is then removed even more efficiently. Following 16 days of exposure to NDMA in the drinking water (10, 30, or 100 ppm), N7MG accumulated in rat liver DNA in a dose-dependent manner, but O^6MG did not (31). Both adducts accumulated in the DNA of mouse liver. This difference was shown to be due to species differences in O^6AT. Administration of 2 mg/kg/day NDMA to rats for 3 weeks did not result in accumulation of O^6MG in hepatocytes but did cause more rapid repair of this adduct (32). The same exposure regimen did not change the concentration of O^6MG in nonparenchmal cells. No molecular dosimetry studies have been conducted on NDMA to provide data comparable to the results now available for NDEA. Thus, our understanding of the mechanisms involved determining the dose-response relationship for NDMA is more limited. The major difference in slope observed for liver cell tumors between NDMA and NDEA may well be related to the large differences in the formation and repair of O^6MG *versus* O^6EG and O^4MT *versus* O^4ET.

Cell replication is another critical factor in carcinogenesis. There may be two reasons for this: (*a*) cell replication before DNA repair is required to convert promutagenic DNA adducts into mutations; and (*b*) clonal expansion of populations of initiated cells increases the probability of additional genetic events. A systematic evaluation of cell proliferation has been reported (33) for the liver of rats exposed to the same NDEA concentrations and times used in the molecular dosimetry study on O^4ET (24). Hepatocellular proliferation was increased in a time and dose related manner. After 10 weeks of exposure to 40 or 100 ppm NDEA, cell proliferation was 800 and 1500% greater than in control rats, respectively. Likewise, exposure to 4 or 10 ppm induced 300 and 400% increases, while 0.4 and 1 ppm did not significantly affect proliferation. Thus, even though the molecular dose of O^4ET was proportional to dose, rats receiving concentrations of NDEA >1 ppm have a greater probability of developing liver cancer. This correlates with the portion of the BIBRA (1, 2) dose-response curve exhibiting the change in slope between low and high dose effects. The bioassay data lack adequate sensitivity to demonstrate whether or not an additional nonlinearity might be associated with saturation of O^4ET repair at even lower doses.

Similar data on cell proliferation are not available for NDMA. No increase in[^{3}H]thymidine incorporation was demonstrated after 16 days of exposure of rats to 10, 30, or 100 ppm NDMA in the drinking water (31) and no increase in metabolic incorporation of ^{14}C from labeled NDMA into adenine was detected following 3 weeks of gastric intubation of 2 mg/kg/day NDMA (32).

Richardson *et al.* (34) examined hepatocyte initiation in rats exposed to NDEA using a protocol similar to previous molecular dosimetry (24) and cell proliferation (33) studies. Initiation was time and dose dependent. Exposures to 4 or 10 ppm NDEA caused increases in γ-glutamyl transferase-positive foci that were dependent on the product of time (t) and concentration (C) for up to 140 days. Exposure to 40 ppm resulted in the rapid formation of foci followed by a plateau in their number,

but a clear increase in size. This was similar to earlier observations using this concentration by Dyroff *et al.* (35) and Richardson *et al.* (36). These data further support the concept that the $C \times t$ relationships will be constant only when the molecular dose is proportional to administered dose and cell proliferation is constant.

The third paper by Gray *et al.* (3) examined the effect of age on tumor induction by NDEA. The greater susceptibility of 3-week-old rats and the lesser susceptibility of 20-week-old rats to NDEA most likely reflects the influence of age dependent cell proliferation. Support for this conclusion stems from the studies of Dyroff *et al.* (35) and Richardson *et al.* (36). The influence of age on NDEA induced hepatic initiation was examined by quantitating the number of γ-glutamyl transferase-positive foci induced by 4 weeks of NDEA administration (40 ppm) to groups of rats that were 4–14 weeks of age at the start of carcinogen exposure. The results demonstrated that the younger rats were 15-fold more susceptible than the older rats to the initiating effects of NDEA. The same study demonstrated that the molecular dose of O⁴ET that accumulated in 4- *versus* 8-week-old rats was similar, although the younger animals reached steady-state concentrations sooner due to greater water consumption per unit body weight. Between 4 and 8 weeks of age, the labeling index of hepatocytes of control rats decreased from ~3.0% to 0.8% (26). Data on 20-week-old rats have not been reported.

A theoretical paper has recently been published that modeled data from single and continuous dose administration of NDEA in the framework of a two-mutation model for carcinogenesis (37). The authors concluded that: (*a*) Predictions of the two-mutation oncogenic model are consistent with empirical data on NDEA-induced hepatocarcinogenesis; (*b*) the probability of the first genetic alteration (initiation) is linearly dependent on applied dose and decays exponentially following a pulse (single) dose or cessation of exposure; (*c*) the probability of initiation is proportional to the number of O⁴ET DNA adducts resulting from NDEA exposure, indicating that these adducts are the likely promutagenic lesions in NDEA-induced hepatocarcinogenesis; (*d*) the mitotic rates of initiated and transformed cells are nonlinear with dose; (*e*) the average growth rate of initiated hepatocytes as a function of NDEA dose is related to Druckery's slope; (*f*) the probability of the second genetic event (transformation) is independent of applied dose, suggesting that it is the result of a spontaneous genetic alteration. This is quite different from the conclusions of Peto *et al.* (1, 2). Further investigation will be needed to help explore the ramifications of these differences.

Current knowledge of NDEA and NDMA carcinogenesis is almost exclusively based on studies of liver. Much less is known about esophagus or other tissues. Likewise, most of our understanding of the mechanism is from research on rats. Little can be said about the experiments on mice and hamsters reported by Gray *et al.* (3). The number of animals used per dose group is small compared to normal bioassay or research protocols. In view of the readily saturated and slow resynthesis of O⁶AT DNA repair activity in hamsters (27), one might have expected the hamster to be more susceptible to NDEA. This hypothesis would be better tested; however, with NDMA, where O⁶MG represents the major promutagenic DNA adduct.

In summary, our present understanding of the mechanisms involved in NDEA and NDMA carcinogenesis suggests that it would be useful to reevaluate the dose-response and age relationships observed in the BIBRA studies (1–3) in terms of the molecular dose of O^6-alkylguanine and O^4-alkylthymine, dose and age related effects on cell proliferation, and the length of carcinogen exposure. Little is known about the contribution of other promutagenic DNA adducts, sites of critical mutations, and the influence of dose on their induction. The BIBRA papers (1–3) provide a valuable resource for developing and testing many important hypotheses regarding these and related issues that are critical for improved understanding of chemical carcinogenesis and, eventually, more accurate assessment of human risk.

Advances in Statistical Methods for Evaluating Carcinogenesis Bioassays

The present studies are of exceptional size, involving over 5000 rodents exposed to 16 concentrations of nitrosoamines and followed for a lifetime (1–3). With all of this high quality data, Richard Peto and his colleagues have provided a *tour de force* of the best statistical methodologies for dealing with survival/incidence data, especially from chronic animal studies. Even with the large amount of data present in these experiments, they show how basic results can be clarified by the use of proper statistical techniques. Some of these results may, without appropriate statistics, appear totally obscure and may even appear to be opposite from what the unanalyzed data suggest. Proper analysis of the data, however, shows smooth and consistent dose response effects for each cancer type among the rodents given the nitrosamine.

The methodology which the authors use requires a determination of whether the animal died due to the tumor of interest or whether the presence of the tumor was "incidental." Some pathologists prefer not having to make this determination, since in some cases it is difficult. In a paper by Portier *et al.* (38), a large set of tumor types was analyzed from the control data of the NTP data base. Generally, most tumors were considered incidental, rather than actual causes of death. This included liver cancer, which is the main focus of the present papers. Brantom and Grasso, who were responsible for the pathology in the present studies (1, 2), estimated that about two-thirds of the liver tumors were a cause of death. This issue was considered also by Lagakos and Ryan (39), who used the ED_{01} study of 2-acetylaminofluorene. A possibly important difference is that in the current studies, animals with palpable liver tumors were sacrificed, *i.e.*, died due to the tumor. This is an issue of particular interest to those developing statistical significance tests. The procedure used by Peto *et al.* (1, 2) is, however, reasonably robust and so the issue is managed fairly adequately in the current setting (see Ref. 39 for additional discussion).

The Portier *et al.* (38) NTP analysis also considered what statistical functions best described "time to tumor." They are in complete agreement with Peto *et al.* (1, 2) in observing that the Weibull distribution is the most appropriate. Since the NTP study considered only control animals, liver cancer could be modeled, whereas esophageal tumors could not be due to their rarity in untreated Fischer rats. With regard to liver tumors, the BIBRA study used the 7th power of time as the Weibull shape parameter. This is somewhat higher than observed with the NTP rats (38). The variability in the data, however, suggests that the two studies are consistent. There is also no reason why two different strains of rats necessarily should exactly agree. It is, however, important to recognize that for time-to-tumor the Weibull distribution is both the distribution of choice across studies and closely related to multistage theories of carcinogenesis.

An unusual feature of the present study is that it involved 16 experimental doses. This must be, if not the largest, then one of the largest dose-response studies ever successfully carried out. The doses cover a range of about 2.5 orders of magnitude (0.033 to 16.896 ppm). What is especially interesting is that measurable increases in tumor rates are seen even at some of the lowest doses, with continuing increases up to the highest dose (*e.g.*, NDEA liver tumors). This wide range of sensitivity is a result of the experimentalists continuing the study until the animals were extremely old and the statistical authors using a median time-to-tumor measure expressed as a Weibull parameter instead of the simpler measure of the proportion of observed tumors. For example, the observed proportion of tumor bearing animals for all liver tumors among NDMA exposed female rats was over 90% for animals in each of the highest 6 exposure groups, showing its insensitivity for detecting further increases, when in fact the Weibull parameter *b* continued to increase with increasing dose. Further, we recognize these nitrosamines as being very potent carcinogens in the sense that at the highest doses, essentially all animals were tumor bearing, with only their time of occurrence changing with dose. This is not often seen with the vast majority of compounds tested today for carcinogenicity.

Both the National Toxicology Program and many governmental regulatory groups in the United States terminate chronic animal studies after 2 years. What effects would a 2-year sacrifice have had on this study? Perhaps the most significant difference would have been the possible loss of information about liver tumors at the lowest doses (See Figs. 6*A, B, C,* and *D* in Ref. 1). Although this is speculation, it is likely that any observed low dose linearity would be lost. In fact, the liver tumors might have been viewed in the same way as the esophageal tumors, namely, consistent with a threshold. It is then a question for the regulatory agencies to decide if they are losing important information by terminating their studies early, especially in view of the many weak carcinogens in limited sized studies with which they are involved. It must be recognized, however, that the BIBRA studies examined a much more limited set of tissues than is routinely examined in conventional bioassays. The effects of marked increases in background tumors seen in many other tissues with increasing age may affect the ability to detect small chemically induced increases in tumors.

An interesting feature of this study is that its size has permitted analysis of the specific cell types among the liver tumors. Differences between the slopes of the individual dose-response functions are important and should not be overlooked when considering extrapolating these findings to very low doses. As discussed above, a greater understanding of dose response would probably be achieved by measuring the molecular dose and cell proliferation in specific target cell types.

The issue of low dose effects is in general very important and the primary points are well illustrated by this study and very well articulated by the authors. First, they point out that the fact that a particular mathematical model fits the observed data is "not strong evidence that it must do so" at lower doses. This is especially relevant with respect to the esophageal tumors and their apparent cubic dose-response relationship. The second principle is that with the presence of background tumors theoretical considerations lead to low dose linearity (40), *i.e.*, to approximate proportionality between the molecular dose and the excess risk as long as the excess risk does not greatly exceed the background risk. This is illustrated in the liver tumor data of both compounds and is described in the fitted Weibull models for the liver tumors. The final point and the most important one for risk assessors is that the study provides reasonably reliable estimates of the effects of very low doses of nitrosoamine only on rats. It does not provide reliable estimates of effects on humans. This is in general the greatest problem confronting the risk assessors and government regulators.

The rapid advances being made in integrating metabolism, pharmacokinetics, DNA repair, cell replication, oncogene activation, and suppressor gene inactivation provide a potential way to bridge this critical gap.

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James A. Swenberg, David G. Hoel and Peter N. Magee

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